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PATENT]

**METHOD OF INHIBITING ANGIOGENESIS AND TUMOR GROWTH
AND PREVENTING TUMOR GROWTH AND METASTASES**

[0001] This invention was made with Government Support under grant #HL19278, awarded by the National Institutes of Health. The government has certain rights in this invention. This is a continuation application of United States Patent Application Serial No. 09/090,757, filed on June 4, 1998.

1. FIELD OF THE INVENTION

[0002] The invention is directed to novel methods of use of a monoclonal antibody which inhibits platelet function and also acts as an antagonist of the integrins GPIIb/IIIa($\alpha_{IIb}\beta_3$) and $\alpha_v\beta_3$. One method of use includes inhibiting angiogenesis and thus angiogenic diseases such as rheumatoid arthritis, psoriasis, diabetic retinopathy, and macular degeneration. Other novel methods of use include preventing and inhibiting tumor growth and metastases.

2. BACKGROUND OF THE INVENTION

2.1. 7E3 and c7E3 Monoclonal Antibodies

[0003] 7E3 is a monoclonal antibody that has been well defined in published studies (Coller, 1985, J. Clin. Invest. 76:101-108 and Coller, 1986, J. Cell Biol. 103:451-456). This antibody has been found to inhibit platelet aggregation via its binding to platelet GPIIb/IIIa ($\alpha_{IIb}\beta_3$) receptors and prevent thrombus formation. It has also been found that 7E3 binds to $\alpha_v\beta_3$ receptors (Marcinkiewicz et al.,

1996, Biochem. J. 317: 817-825) and prevents the attachment and spreading of endothelial cells on extracellular matrix protein ligands for $\alpha_v\beta_3$ (Tam et al., 1998, Circulation, in press and Varner et al., 1997, Thromb. Haem. 77(Suppl.) 158).

[0004] 7E3 is the forerunner of the murine/human chimeric antibody Fab fragment (c7E3 Fab; abciximab; ReoPro™) that is approved for human use in the prevention of ischemic complications of percutaneous coronary interventions. C7E3 Fab has also been found to bind to both GPIIb/IIIa ($\alpha_{IIb}\beta_3$) and $\alpha_v\beta_3$ receptors (Reverter et al., 1996, J. Clin. Invest. 98:863-874 and Nakada et al., 1997, J. Amer. Col. Cardiol. 29:243A).

2.2. Angiogenesis

[0005] Angiogenesis is the development of new capillaries from preexisting blood vessels (reviewed in Weinstein-Saslow, 1994, The FASEB Journal 8:402-407 and Folkman et al.; 1987, Science 235:442-447). A number of factors are capable of stimulating an angiogenic response. Examples include aFGF, bFGF, TGF α , TGF β , EGF, and angiogenin. Some of these factors act directly on endothelial cells, whereas others activate local inflammatory cells to induce angiogenesis. Angiogenesis can have both beneficial and detrimental effects. It can, on the one hand, facilitate wound healing. However, on the other hand it may cause such inflammatory diseases as rheumatoid arthritis, macular degeneration, psoriasis, and diabetic retinopathy.

2.3. Tumor Growth and Metastases

[0006] The invasive growth and widespread dissemination of human solid tumor cancers remains a problem of great clinical significance. Novel clinical therapies that inhibit tumor proliferation, invasion, and metastasis could improve the outcome of solid tumor cancers.

[0007] Tumor-associated angiogenesis is a potential target for such therapy since it has been implicated not only in the growth of tumors but also in their metastasis (Liotta et al., 1991, Cell 64:327; Weinstat-Saslow et al., 1994, FASEB J 8:401; Blood et al., 1990, Biochim. Biophys. Acta 1032:89; Folkman, 1992, Semin. Cancer Biol. 3:65; and Weidner et al., 1991, N. Engl. J. Med. 324:1). Tumor-induced angiogenesis is initiated by growth factors and cytokines that are released from the tumor or from inflammatory cell infiltrates (Brown et al., 1993, Am. J. Path. 143:1255; Brown et al., 1995, Human Path. 26:86; Leek et al., 1994, J. Leukocyte Biol. 56:423; Hatva et al., 1995, Am. J. Pathol. 146:368; and Plate et al., 1992, Nature 359:845). Growth factors and cytokines which are expressed by tumor cells stimulate angiogenesis in a number of animal models including the chick chorioallantoic membrane model, the corneal pocket angiogenesis model, and models involving spontaneous and xenotransplanted tumor growth (Brooks et al., 1994, Cell 79:1157; Brooks et al., 1994, Science 264:569; Brooks et al., 1995, J. Clin. Invest. 96: 1815; and Friedlander et al., 1995, Science 27:1500).

[0008] Recently, a number of agents have been found to inhibit angiogenesis and tumor growth. Examples include endostatin (O'Reilly et al., 1997, Cell

However, mixed results have been obtained with anti-platelet aggregating agents such as aspirin (summarized in Karpatkin et al., 1988, J. Clin. Invest. 81:1012-1019). It has been hypothesized that platelets appear to contribute to metastases by their adhesive interaction with tumor cells via the adhesive proteins fibronectin and von Willebrand factor (Karpatkin et al., 1988, J. Clin. Invest. 81:1012-1019).

[0011] As described above, substances known in the art either inhibit angiogenesis and primary tumor growth or prevent metastases. Therefore, it is necessary to administer one course of treatment to inhibit the growth of the primary tumor and another course of treatment to prevent metastases. It would be useful if there was a substance that acts to inhibit angiogenesis and tumor growth as well as prevents metastases. Therefore, it is an object of the invention to formulate a substance that can be used for both purposes.

3. SUMMARY OF THE INVENTION

[0012] The invention is directed to methods for inhibiting angiogenesis and tumor growth as well as preventing tumor growth or metastases in a mammal in need thereof comprising administering to said mammal a monoclonal antibody or fragment thereof which acts as an antagonist of the integrins GPIIb/IIIa ($\alpha_{IIb}\beta_3$) and $\alpha_v\beta_3$ in an amount effective to inhibit angiogenesis or tumor growth or in an amount effective to prevent tumor growth or metastases in said mammal. The fragment may be an Fab, Fab' or (Fab')₂ fragment or derivative thereof (e.g.,

chimeric, humanized, etc.). The mammal is preferably a primate or dog or cat and most preferably a human patient.

[0013] In a specific embodiment, the monoclonal antibody has the identifying characteristics of 7E3 which is produced by the ATCC 8832 hybridoma cell line. Alternatively, the monoclonal antibody may be a chimeric mouse/human monoclonal antibody which comprises a variable or antigen binding region of non-human origin and a constant region of human origin and has the identifying characteristics of the monoclonal antibody, c7E3.

4. **BRIEF DESCRIPTION OF THE FIGURES**

[0014] **Figure 1: Human tumor culture in human skin/SCID mouse model.**

In the human skin/SCID mouse model, six week old SCID mice were engrafted with an 8 mm x 13 mm piece of human neonatal foreskin. Four weeks later, the human skin was inoculated with three million human M21L melanoma cells, which were deficient in the expression of the integrin, $\alpha_v\beta_3$. After two weeks, mice with palpable tumors were treated either by intravenous or intraperitoneal injections three times per week for three weeks with antibodies or saline. Tumors were excised and analyzed for tumor size and weight.

[0015] **Figure 2A-(1-3): Human tumor angiogenesis in the human skin/SCID mouse model.** Tumors were removed from human skin grafts bearing tumors, embedded in OCT and snap frozen. Five micron cryostat sections of tumors were fixed in acetone and stained by indirect immunofluorescence with

anti-human CD31 (PECAM; **Figure 2A-2**), a marker of endothelial cells (red) or with anti-von Willebrand factor (**Figure 2A-1**), a marker of human and mouse blood vessels (green). Colocalization of von Willebrand factor and human CD31 can be observed by merging the two images (yellow; **Figure 2A-3**). Blood vessels which are positive for both markers are of human origin (arrows).

[0016] **Figure 3A-B: 7E3 inhibits human tumor growth in human skin/SCID mouse model.** Six week old SCID mice were engrafted with an 8 mm x 13 mm piece of human neonatal foreskin. Four weeks later, the human skin was inoculated with three million human M21L melanoma cells. After two weeks, mice with palpable tumors were treated by intravenous or intraperitoneal injections three times per week for three weeks with 300 µg doses of 7E3, AP3 (an isotype matched control antibody) or saline. Tumors were excised and analyzed for tumor size (**Figure 3A**) and weight (**Figure 3B**).

[0017] **Figure 4A-B: 7E3 inhibits human tumor growth in human skin/SCID mouse model as well as LM609.** Six-week old SCID mice were engrafted with an 8 mm x 13 mm piece of human neonatal foreskin. Four weeks later, the human skin was inoculated with three million human M21L melanoma cells. After two weeks, mice with palpable tumors were treated either by intravenous or intraperitoneal injections three times per week for three weeks with 300 mg doses of 7E3, LM609 (an anti- $\alpha_v\beta_3$ specific monoclonal antibody), an isotype-matched control anti- β_3 antibody or saline. Tumors were excised and analyzed for tumor volume (**Figure 4A**) and weight (**Figure 4B**).

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[0018] **Figure 5: Both 7E3 and LM609 significantly inhibit tumor growth.**

A comparison of tumor volumes and weights for three separate experiments are shown. Using the exact Wilcoxon rank-sum test, stratifying on experimental groups, both the LM609 and 7E3 treated groups had smaller tumor volumes ($p=0.003$ and $p<0.0001$, respectively) and tumor weights ($p=0.02$ and $p=0.0007$, respectively) than control treated animals.

[0019] **Figure 6(A-1-3), (B1-3),(C): 7E3 and LM609 inhibit human angiogenesis.** Tumors treated with saline, 7E3 or LM609 were removed from the human skin grafts, embedded in OCT and snap frozen. Five micron cryostat sections were fixed in acetone and stained by indirect immunofluorescence with anti-human CD31 (**A-1 = saline treated; A-2 = LM609 treated; and A-3 = 7E3 treated**) or with anti-murine CD31 (**B-1 = saline treated; B-2 = LM609 treated; and B-3 = 7E3 treated**). Tissue sections were photographed at a magnification of 200 X (C). Blood vessels staining positive for human CD31 were quantified on 15 randomly selected microscopic fields at 200X.

5. DETAILED DESCRIPTION OF THE INVENTION

5.1. Preparation of Monoclonal Antibodies

[0020] The monoclonal antibody used in the method of the present invention may be prepared using procedures known in the art.

[0021] In a specific embodiment, the monoclonal antibody has the identifying characteristics of 7E3. The procedure described in U.S. Patent No. 5,440,020 may

be used to obtain said monoclonal antibody. Specifically, human blood platelets are injected into mice. The mouse spleen is removed and fused with mouse myeloma cells by a modification of the technique of Levy et al., 1984, Curr. Top. Microbiol. Immunol. 81: 164. The fused cells are incubated and then further incubated in HAT medium. The cells are then diluted out and screened in a screening assay for antifibrinogen receptor activity (Coller et al., 1983, J. Clin. Invest. 72:3235-338). There is selected a clone which reacts with normal human blood platelets and with dog blood platelets, fails to react with thrombasthenia platelets or human platelets whose GPIIb/IIIa complex was dissociated with EDTA, reacts slowly with unactivated human platelets and more rapidly with ADP activated human platelets and blocks the interaction of fibrinogen with platelets induced by ADP (Coller et al., 1983, J. Clin. Invest. 72:325-338). Additionally, the antibody acts as an antagonist to the integrin $\alpha_v\beta_3$ by inhibiting the binding of extracellular matrix ligands to integrin $\alpha_v\beta_3$ and preventing the $\alpha_v\beta_3$ -dependent attachment of cells to extracellular matrix protein ligands. The binding of the antibody to $\alpha_v\beta_3$ receptors may be detected, for example, by platelet adhesion assays as described in Coller et al., 1991, Blood 77:75-83. The antibody is subsequently isolated from the supernatant in wells or flasks.

[0022] Alternatively, the monoclonal antibody may be obtained from a hybridoma having the identifying characteristics of the ATCC HB 8832 hybridoma cell line. The hybridomas are injected intraperitoneally into Pristane pretreated BALB/c mice and the antibodies are isolated from the ascitic fluid.

heavy chain constant region. The light chain comprises an antigen binding region derived from the light chain of the non-human antibody linked to a human light chain constant region.

[0025] This antibody may be obtained using procedures known in the art (see, for example, PCT appln no. PCT/US94/12779). Specifically, the chimeric antibodies are produced by preparing for each of the light and heavy chain components a fused gene comprising a first DNA segment that encodes at least the functional portion of the platelet-specific variable region of nonhuman origin linked to a second DNA segment encoding at least a part of a human constant region. Each fused gene is assembled in or inserted into an expression vector. Host cells are then transfected with the expression vector. The transfected host cells are cultured under conditions which promote expression of the DNA sequences encoding the chimeric antibody and the expressed antibody is recovered.

[0026] In a preferred embodiment, an Fab fragment is used. Said fragment may be purchased from Centocor and goes by the tradename ReoPro™ or generated using procedures described *supra*.

5.2. Compositions and their Uses

[0027] The monoclonal antibody described *supra* can be used to inhibit angiogenesis and thus treat inflammatory diseases which may include but are not limited to rheumatoid arthritis, diabetic retinopathy, psoriasis, and macular degeneration. Additionally, said monoclonal antibody can be used to inhibit and

prevent tumor growth, and prevent metastases. The individual to be treated may be any mammal and is preferably a primate, a dog or cat and most preferably a human patient. The amount of monoclonal antibody administered will vary according to the purpose it is being used for and the method of administration. Administration may also be oral or by local injection into a tumor or tissue. In a preferred embodiment, the monoclonal antibody is administered intravenously. Generally, the dosage range is 0.25 mg/kg as a bolus dose. This may be followed by a continuous infusion of 0.125 mg/kg/min for about 3 weeks.

[0028] Alternatively, DNA encoding preferably a fragment of said monoclonal antibody may be isolated from hybridoma cells and administered to a mammal. The DNA may be administered in naked form or inserted into a recombinant vector, e.g., vaccinia virus.

[0029] The monoclonal antibody used in the method of the present invention may be formulated by any of the established methods of formulating pharmaceutical compositions, e.g. as described in Remington's Pharmaceutical Sciences, 1985. For ease of administration, the monoclonal antibody will typically be combined with a pharmaceutically acceptable carrier. Such carriers include water, physiological saline, or oils. Except insofar as any conventional medium is incompatible with the active ingredient and its intended use, its use in any compositions is contemplated.

6. EXAMPLE

6.1. Materials and Methods

6.1.1. Antibodies and Reagents

[0030] Murine 7E3, was produced at Centocor, Malvern, PA. LM609 was the kind gifts of Dr. David Cheresch. AP3 was a gift from Peter Newman, Blood Research Institute, Milwaukee, WI. Anti-human CD31 was purchased from Chemicon International (Temecula, CA). Anti-murine CD31 was purchased from Pharmingen (San Diego, CA). Anti-von Willebrand factor was purchased from Biogenex (San Ramon, CA). Cross-absorbed secondary antibodies were purchased from Chemicon International or from Biosource International (Camarillo, CA). Six-week old CB17 female SCID mice were purchased from Charles River. Fresh human neonatal foreskins were obtained from the Cooperative Human Tissue Network of the National Institutes of Health and were stored in RPMI-1640 media supplemented with 2% fetal bovine serum and 1% gentamicin. Human M21L melanoma cells (Fielding-Habermann et al., 1992, J. Biol. Chem. 267:5070-507) were a gift from Dr. David Cheresch. OCT embedding medium was obtained from Baxter (McGraw Park, IL). Fluoromount embedding medium was purchased from Southern Biotechnology Associates (Birmingham, AL).

6.1.2. Cell Culture

[0031] M21L human melanoma cells which were deficient in $\alpha_v\beta_3$ were routinely cultured in RPMI medium (Irvine Scientific, Irvine, CA) supplemented

[illegible]

6.1.3. SCID Mouse Model of Human Tumor Growth

[0032] In this model, tumors were primarily supplied by human blood vessels derived from the human skin, but large tumors eventually recruit some murine blood vessels. This model has the further advantage of essentially excluding the possibility that 7E3 may be working through its antiplatelet effects since 7E3 does not react with murine platelets.

[0033] Engraftment of SCID mice with human skins was performed as described (Brooks et al., 1995, J. Clin. Invest. 96: 1815). The general procedure used is shown in Figure 1. Six-week old SCID mice were engrafted with an 8 mm x 13 mm piece of human neonatal foreskin. Four weeks later, the human skin was inoculated with three million human M21L melanoma cells. After two weeks, mice with palpable tumors were treated either by intravenous or intraperitoneal injections three times per week for three weeks with 50 μ l of saline or 300 μ g doses of 7E3, LM609 (an anti- $\alpha_v\beta_3$ murine monoclonal antibody) or an isotype-matched control murine monoclonal antibody. At the end of the three week treatment period, tumors were excised and analyzed for tumor size using

calipers and for wet weight. Tumor volumes were calculated using the formula $(w^2 \times l)/2$ (w is width and l is length).

6.1.4. Immunohistochemistry and Quantification of Blood Vessels

[0034] Tumors were removed from human skin grafts bearing M21L tumors, embedded in OCT and snap frozen. Five micron cryostat sections of tumors were fixed for one minute in acetone and stained by indirect immunofluorescence by incubating sections in 2% bovine serum albumin in saline for two hours followed by incubation of sections in 5 µg/ml of murine anti-human CD31 (PECAM), rat anti-murine CD31, or rabbit anti-von Willebrand factor. Sections were washed five times with saline and incubated for 1 hour in goat anti-rabbit-IgG-FITC or goat anti-mouse-IgG-rhodamine. Sections were washed five times with saline and mounted with Fluoromount. Two color staining for colocalization of von Willebrand factor and human CD31 was performed essentially as described (Brooks et al., 1995, J. Clin. Invest. 96: 1815). Blood vessels staining positive for human CD31 were quantified on 15 randomly selected microscopic fields at 200X.

6.1.5 Statistical Analysis

[0035] Statistical analyses were performed using StatXact 3 for Windows, CYTEL software Corporation, Cambridge, MA.

6.2. Results

6.2.1. Growth of Human Melanoma in the SCID Mouse/Human Skin Model of Human Angiogenesis

[0036] To assess whether M21L tumors were supplied by human blood vessels derived from the human skin or mouse blood vessels infiltrating the human skin graft, control tumors were excised four weeks after inoculation of tumor cells (see Figure 1) and frozen sections from these tumors were stained with an antibody specific for human CD31 (PECAM), a constitutive marker of adult endothelial cells (DeLisser et al, 1994, Immunol. Today 15:490-495) and with an antibody directed against both mouse and human von Willebrand factor, an extracellular matrix protein marker of mature blood vessels. The majority of the blood vessels identified by either hematoxylin and eosin staining or by immunofluorescence analysis were associated with the edges of nests of M21L tumor cells (Figure 2A-(1-3)). Blood vessels with both large and small lumens stained positively for von Willebrand factor (**Figure 2A-1**); vessels with small lumens were positive for human CD31 (**Figure 2A-2**). When the anti-CD31 and anti-von Willebrand factor images were merged to identify the overlap in expression of these two markers (seen as yellow; **Figure 2A-3**), the majority of the vessels were shown to be positive for both human CD31 and von Willebrand factor expression (arrows). Approximately 10% of the vessels were positive only for von Willebrand factor. Since the antibody against von Willebrand factor reacts with both mouse and human blood vessels, these results indicate that the majority of the blood vessels in the tumor grown in the human skin graft are of human origin, but ~10% are of

murine origin.

6.2.2. 7E3 Inhibits Tumor Growth

[0037] To evaluate the effect of 7E3 on human angiogenesis and tumor growth, tumor-bearing mice were injected either intravenously or intraperitoneally three times a week for three weeks with 300 µg of 7E3, a control isotype-matched anti-integrin β_3 antibody, AP3 or with saline (Figure 3A-B). While both 7E3 and AP3 react with integrins $\alpha_v\beta_3$ and $\alpha_{IIb}\beta_3$, only 7E3 inhibits the function of these integrins. Mice were treated three times a week for three weeks with 300 µg doses of each antibody. Tumor dimensions were measured with calipers at the end of the three weeks of treatment. Tumors were then resected and wet weights determined. Systemic administration of 7E3 resulted in a significant reduction in both tumor weight and tumor volume when compared to control treatments (Figure 3A-B). Tumors from 7E3 treated mice were 60% smaller on the basis of tumor weight (Figure 3B) and 64% smaller on the basis of tumor volume (Figure 3A) when compared to tumors treated with the control antibody AP3. 7E3 treatment resulted in a 72% reduction in tumor weight (Figure 3B) and a 75% reduction in tumor volume (Figure 3A) when compared to tumors from saline treated mice.

6.2.3. 7E3 Prevents Tumor Growth as well as L609

[0038] To further evaluate the efficacy of 7E3 to inhibit tumor growth, the potency of 7E3 was compared to that of LM609, a specific monoclonal antibody antagonist of integrin $\alpha_v\beta_3$ with demonstrated anti-angiogenic properties (Brooks

et al., 1994, Cell 79:1157; Brooks et al., 1994, Science 264:569; Brooks et al., 1995, J. Clin. Invest. 96: 1815; and Friedlander et al., 1995, Science 27:1500). Thrice weekly intravenous or intraperitoneal injections of 300 µg of 7E3, 300 mg of LM609 or of an equivalent volume of saline were administered to groups of mice bearing palpable tumors normalized for size. As seen in Figure 4A-B, 7E3 was as effective as LM609 in inhibiting tumor growth in the SCID mouse/human skin model. In this experiment, 7E3 and LM609 inhibited tumor volumes by 85% and 82% respectively (**Figure 4A**), and tumor weights by 64% and 60% respectively (**Figure 4B**). Studies comparing 7E3 to LM609 were performed three times with six to eight mice per treatment group. In each experiment, treatment of mice bearing tumors with either 7E3 or LM609 resulted in statistically significant suppression of tumor growth as evaluated either by tumor volume or tumor weight (Figure 5). Using the exact Wilcoxon rank-sum test, stratifying on experimental groups, both the LM609-treated and 7E3-treated animals had significantly smaller tumor volumes than did the saline control group ($p=0.003$ and $p<0.0001$). Similar results were obtained using tumor weights instead of tumor volumes. Both the LM609-treated and 7E3 treated animals had significantly lighter weight tumors than the saline-treated animals ($p=0.02$ and $p=0.0007$).

6.2.4. **7E3 Inhibits Tumor-Induced Angiogenesis**

[0039] Histological analysis of 7E3 treated tumors demonstrated that 7E3 significantly inhibits human angiogenesis. As shown in Figure 6A(1-3), tumors

from mice treated with either saline or control IgG have numerous human CD31 positive blood vessels. Tumors from mice treated with 7E3 or LM609 exhibit a significant reduction in human CD31 positive blood vessels. When human CD31 positive vessels were quantified by counting the number of vessels per high power (200X) microscopic field in fifteen representative microscopic fields, an average of 70 vessels per field were observed in saline treated tumors. In contrast, treatment with 7E3 resulted in an average of 33 vessels per section and LM609 resulted in an average of 23.5 vessels per microscopic field (Figure 6[B]C).

[0040] When tumor sections were stained for the presence of murine blood vessels by staining frozen sections with an antibody that is specific for murine CD31 (**Figure 6B(1-3)**), significantly fewer murine vessels were present in 7E3- and in LM609- treated tumors than in control-treated tumors (Figure 6C). Since 7E3 and LM609 do not react with murine integrins, these results suggest that in this model, angiogenesis proceeds in two phases, the first in which human blood vessels supply the tumor, and the second, in which the large tumor can recruit blood vessels from the surrounding murine tissues. By inhibiting the first phase of angiogenesis dependent on the human blood vessels, 7E3 and LM609 prevent the tumor from growing to a size capable of recruiting murine blood vessels.

[0041] The invention described and claimed herein is not to be limited in scope by the specific embodiments herein disclosed, since these embodiments are intended as illustrations of several aspects of the invention. Any equivalent

